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Nitrifying Bacteria in Wastewater Reservoirs

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Deep wastewater reservoirs are used throughout Israel to store domestic wastewater effluents for summer irrigation. These effluents contain high concentrations of ammonia (≤5 mM) that are frequently toxic to photosynthetic microorganisms and that lead to development of anoxic conditions. Population dynamics of nitrifying bacteria and rates of nitrification were studied in two wastewater reservoirs that differed in organic load and degree of oxygenation and in the laboratory under controlled conditions, both by serial dilutions in mineral medium and microscopically with fluorescein isothiocyanate-conjugated antibodies prepared against local isolates. The difference in counts by the two methods was within 1 order of magnitude. In the laboratory, an O_2 concentration of 0.2 mg liter^{-1} was close to optimal with respect to growth of NH_3 oxidizers on domestic wastewater, while O_2 concentrations of 0.05 mg liter^{-1} supported significant rates of nitrification. It was found that even hypertrophic anaerobic environments such as the anaerobic hypolimnion of the wastewater reservoir or the anaerobic settling ponds are capable of sustaining a viable, although not actively nitrifying, population of *Nitrosomonas* spp. and *Nitrobacter* spp., in contrast to their rapid decline when maintained anaerobically in mineral medium in the laboratory. Nitrification rates of NH_3 in effluents during storage in the reservoirs were slower by 1 to 2 orders of magnitude compared with corresponding rates in water samples brought to the laboratory. The factors causing this inhibition were not identified.

Effluents of domestic wastewater treatment plants in Israel are stored in large, deep (6 to 10 m) reservoirs. These effluents are collected during the winter and used for irrigation during the dry season. The quality of these waters is variable, from high-quality effluents after tertiary treatment to partially treated or untreated sewage. In the latter case, the reservoir itself becomes part of a treatment setup. It is possible to maintain a stable aerobic steady state in such a water body only if biological oxygen demand (BOD) is low and photosynthesis is not restricted. NH_3 concentrations in domestic wastewater, however, are frequently inhibitory to photosynthesis (4) and should be kept at a minimum, together with respiration rates (1, 2), to avoid development of anoxic conditions. Conventional wastewater treatment is rather inefficient in removing NH_3, and 50 to 70% of the initial concentration of NH_3 remains in the effluents unless very long retention times (in the case of stabilization ponds) or an expensive tertiary treatment stage is employed for this purpose (4, 12, 21). If not, a better understanding of the factors controlling NH_3 elimination via microbial nitrification is needed before a nuisance-free maintenance regime can be devised for deep wastewater reservoirs and stabilization ponds. In a previous study (3) I noticed that there exists a small but significant population of nitrifying bacteria (10^2 to 10^3 cells of *Nitrosomonas* sp. and *Nitrobacter* sp. per ml) in water samples from a wastewater reservoir as well as from the preceding overloaded anaerobic stabilization ponds. The problem of how to maintain optimal nitrification rates in such a biotope is not only a question of the presence or the absence of aerobic conditions. The factors that determine distribution and activity of nitrifiers in organically overloaded biotopes are not clear, and although considered obligate aerobes they are occasionally found in microaerophilic (14) or even anaerobic environments (9).

In this study I describe the distribution of nitrifying bacteria in two situations: (i) an overloaded stabilization pond system with an attached wastewater reservoir that receives low-quality effluents, and (ii) a reservoir that receives high-quality effluents. The relations of the presence and activity of nitrifying bacteria to the oxygenation state of these biotopes were studied in the field and in the laboratory under controlled conditions.

MATERIALS AND METHODS

Sampling and observation sites. Two sites were chosen for comparing the distribution of nitrifying bacteria in wastewater reservoirs. The Beer Sheba treatment plant is an overloaded wastewater treatment system that is based on a series of stabilization ponds with a collecting reservoir at the end (Ram Reservoir). This reservoir has a surface area of 8 hectares; it is 6 m deep and is operated at a high turnover rate with a variable retention time of 20 to 40 days, with an average retention time of 20 days for the water at the sampling point used during this study. Because of its high turbidity (compensation point at 25 to 50 cm), it is thermally stratified throughout the summer, with a stable thermocline being established at 200 cm (2). Most of its volume is anaerobic for most of the time because of the high oxygen demand of the effluents it receives. In Table 1 the water quality parameters of this facility are summarized.

The Maale Kishon Reservoir receives effluents of the highest available quality from the wastewater treatment plant in Haifa (activated sludge and trickling filter). This reservoir has a surface area of 1.3 km^2, and its depth during the study was 5 to 6 m. It is divided into two parts, with water flowing into the northern part and out from the southern part. The two parts are connected by a narrow channel. Typical water quality figures for 1985 for the center of the northern part of the Maale Kishon Reservoir (in milligrams per liter) (Y. Eren, Mekoroth Ltd., personal communication) are as follows: total chemical oxygen demand (COD), 69 ± 10; filtered COD, 56 ± 9; total BOD, 6 ± 2; filtered BOD, 4 ± 0.6; NH_3, 42 ± 16; PO_4^-, 28 ± 13. Both parts are totally mixed and oxygenated throughout the year.

Isolation and culture of ammonia- and nitrite-oxidizing bacteria. Enrichment cultures were innoculated with

TABLE 1. Typical water quality parameters of Beer Sheba wastewater treatment plant[a]

	COD (mg · liter^{-1})		BOD (mg · liter^{-1})		NH_3 concn (mg · liter^{-1})	Chlorophyll *a* concn (mg · liter^{-1})	pH	O_2 concn (mg · liter^{-1}) at noon (surface)
	Total	Filtered[b]	Total	Filtered				
Raw sewage	1,040 ± 80	480 ± 80	230 ± 70	112 ± 27	70 ± 8	0	8.0 ± 0.1	
Settling pond	1,253 ± 460	560 ± 230	246 ± 25	180 ± 30	71 ± 5	0	7.2 ± 0.1	0
Anaerobic pond	1,200 ± 550	550 ± 220	206 ± 59	130 ± 14	66 ± 3	0.1	7.7 ± 0.1	0
Facultative pond	760 ± 198	480 ± 220	195 ± 25	146 ± 17	65 ± 3	0.8 ± 0.3	7.9 ± 0.1	0.6 ± 0.2
Ram Reservoir	530 ± 99	290 ± 99	125 ± 25	81 ± 6	38 ± 12	0.9 ± 0.2	8.2 ± 0.1	7.8 ± 5.2

[a] For 1979 to 1984; 15 to 30 samples every year.
[b] Samples were filtered through a cellulose nitrate filter (pore size, 0.45 μm).

water samples from two oxidation ponds and a wastewater reservoir and maintained for 2 months in the following medium (in grams per liter): Na_2HPO_4, 13.5; KH_2PO_4, 0.7; $MgSO_4 \cdot 7H_2O$, 0.1; $NaHCO_3$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.18; $FeCl_3 \cdot 6H_2O$, 0.014; 0.5 g of $(NH_4)_2SO_4$ or $NaNO_2$ (sterilized separately) was also included. A serial dilution was then prepared for the enrichment culture and maintained (in the same medium) for 1 month. From the last tube showing nitrification, serial dilutions were prepared, and samples were transferred to silica gel plates prepared by the method of Funk and Krulwich (13) with the same medium. Plates were incubated for 6 weeks, single colonies were transferred to enrichment medium and incubated for 4 weeks, and the serial dilutions were again plated on silica gel. Single colonies were picked and cultured for mass culture and antibody preparation. I considered the cultures axenic because they showed no growth of heterotrophic bacteria upon plating on nutrient agar.

After isolation the following medium was used for all routine cultures of *Nitrosomonas* sp. (in grams per liter): $(NH_4)_2SO_4$, 3.0; K_2HPO_4, 0.5; $MgSO_4$, 0.05; $CaCl_2$, 0.004; chelated iron (sequestren 138; 0.1 mg of Fe) and cresol red (0.05 mg) were also included. The pH was maintained at 8.2 to 8.4 with a sterile K_2CO_3 solution.

Antibody preparation. Antibodies were prepared against whole cells in wild-type rabbits (purchased from a local farm) by using a culture of ammonia or nitrite oxidizers, grown 4 weeks to 20 mg of protein liter^{-1}, concentrated to 2 ml and dispersed in an equal volume of complete Freund adjuvant, and administered subcutaneously to two rabbits. Preparations of three isolates each of ammonia and nitrite oxidizers that were picked randomly from the silica plates were used to inoculate 12 rabbits, and after 6 weeks an adjuvant-free preparation was administered as a booster (2 mg of protein per rabbit). Two weeks later the rabbits were bled, and six weeks later they were given another booster shot and bled again 2 weeks later. Globulins were sedimented in $(NH_4)_2SO_4$, suspended in phosphate-buffered saline, and dialyzed twice against phosphate-buffered saline to eliminate NH_4^+ ions. The antibodies were conjugated with fluorescein isothiocyanate (FITC) by the procedure described by Meynell and Meynell (19). The labeled antibodies were separated from the extra FITC on a G-25 Sephadex column and stored at −80°C. The antibody preparations were tested for cross-reactivity and specificity. All three NH_3-oxidizing isolates cross-reacted, and therefore, only one preparation was used for counting NH_3-oxidizing bacteria in field samples. Morphologically the cells resembled *Nitrosomonas europaea*, but this serotype differed from *N. europaea* ATCC 19718, as it did not react with the antibodies obtained in this study. Of the NO_2^- oxidizers, two serotypes were isolated, and therefore, antibodies against both serotypes were used for counting NO_2^- oxidizers in field samples. Both had the typical pear shape of *Nitrobacter* spp. No further attempt was made to identify these isolates. The optimal titer for fluorescence work with field samples was found to be 1:50 to 1:80, as undiluted antibody reacted nonspecifically. None of the diluted antibodies reacted with any of several *Escherichia coli* strains or with any of several heterotrophic, unidentified bacteria isolated from the stabilization ponds, nor did an antibody preparation from control rabbits react with any isolate of the nitrifying bacteria from the wastewater oxidation ponds and the reservoir.

Counts of nitrifying bacteria. Counts of viable nitrifiers present in water samples collected in the field or in laboratory experiments were performed both by determining NO_2^- and NO_3^- in serial 10-fold dilutions in duplicate samples and microscopically by using fluorescent antibodies. Test tubes were incubated for 21 days at 20°C and then tested for the presence of NO_2^- and NO_3^-. For direct microscopy of field samples, 1-μl water samples were placed on glass slides and heat fixed. Usually, I could not concentrate samples from the field because this obscured fluorescence due to the presence of a high concentration of particulate organic matter. Therefore, unless relatively clean water samples were available, the limit of detection was 1 cell per 1 μl, and lower numbers could be detected only by counts based on serial dilutions. In the case of the Maale Kishon Reservoir, I was able to concentrate water samples 10 times. Autofluorescence of photosynthetic bacteria prevented accurate counting; therefore, after fixation the slides were treated with a hot mixture of acetone-methanol (1:1) to decrease the interfering fluorescence from the photosynthetic bacteria that were abundant in these biotopes. This treatment did not affect counts of nitrifiers in controlled experiments with pure cultures. Autofluorescence of photosynthetic microorganisms was also reduced by counterstaining with 1% acid fuchsin before FITC-conjugated antibody was applied. Observation of the stained slides did not reveal clusters of nitrifiers, which is commonly believed to be the reason for the low number of these bacteria found in counts based on serial dilutions. Both methods of counting nitrifying bacteria practiced in this study have their limitations; and although the difference in the counts was usually within 1 order of magnitude, accuracy of the counts could not be verified.

Operation of chemostat fed by wastewater. A 10-liter chemostat was operated for 12 months continuously; it was fed with sewage from the first anaerobic pond in the Beer Sheba treatment plant as the sole nutrient supply. Variable dilution rates were maintained by a variable-speed peristaltic pump. Oxygen was supplied either by algal photosynthesis regulated by the duration of illumination or by bubbling through air or N_2 at variable rates. The culture was constantly stirred

TABLE 2. Number of nitrifying bacteria in Beer Sheba treatment plant determined with FITC-conjugated antibody

Date (1984)	No. of nitrifying bacteria/ml in:							
	Settling pond		Anaerobic pond		Facultative pond		Reservoir[a]	
	NH_3 oxidizer	NO_2^- oxidizer	NH_3 oxidizer	NO_2^- oxidizer	NH_3 oxidizer	NO_2^- oxidizer	NH_3 oxidizer	NO_2^- oxidizer
8 May	$<1 \times 10^3$	4×10^3	7×10^3	$<1 \times 10^3$	$<1 \times 10^3$	8×10^3	2×10^4	$<1 \times 10^3$
13 May	4×10^3	8×10^3	$<1 \times 10^3$	$<1 \times 10^3$	7×10^3	5×10^3	3.6×10^4	1.9×10^3
22 May	1.4×10^4	3×10^3	1.7×10^4	3×10^3	1.2×10^4	3×10^3	2.6×10^4	4×10^3
28 May	1×10^3	$<1 \times 10^3$	ND[b]	$<1 \times 10^3$	4×10^4	$<1 \times 10^3$	2×10^4	$<1 \times 10^3$
26 June	ND	ND	6×10^3	$<1 \times 10^3$	ND	ND	$<1 \times 10^3$	$<1 \times 10^3$
4 July	6×10^3	$<1 \times 10^3$	2×10^3	$<1 \times 10^3$	2×10^3	$<1 \times 10^3$	$<1 \times 10^3$	$<1 \times 10^3$
7 August	7×10^3	1×10^3	1.2×10^4	2×10^3	4×10^4	2×10^3	6×10^3	3×10^3
10 September	ND	ND	ND	ND	ND	ND	$<1 \times 10^3$	$<1 \times 10^3$
16 September	6×10^3	1×10^3	3×10^3	$<1 \times 10^3$	5×10^3	$<1 \times 10^3$	2×10^3	$<1 \times 10^3$
2 October	ND	ND	ND	ND	ND	ND	3×10^3	1×10^3
6 November	5×10^3	5×10^3	3×10^3	3×10^3	1×10^4	2×10^3	3×10^4	1×10^3

[a] Sampled at 5 cm below the surface at 7:00 a.m.
[b] ND, Not determined.

with a magnetic stirrer. O_2 was monitored with an O_2 meter (model 58; Yellow Springs Instrument Co., Yellow Springs, Ohio) connected to an expanded scale recorder (Goertz Servogor 210). This setup enabled reproducible measurements down to very low O_2 concentrations (0.01 to 0.2 mg of O_2 liter^{-1}). The population size of nitrifying bacteria was determined as described above.

Mass culture of *Nitrosomonas* sp. Axenic mass cultures of *Nitrosomonas* sp. were carried in a 7.5-liter fermentor (Magnaferm; New Brunswick Scientific Co., Inc., Edison, N.J.) operating in a semicontinuous mode with an automatic pH controller set at 8.0 (pH was adjusted with a 20% K_2CO_3 solution) and aerated at 5 liter min^{-1} with air supplemented with 1% CO_2. Anaerobic experiments were performed in a Forma anaerobic work system. All reagents used for anaerobic experiments were flushed for 30 min in argon before they were introduced into the anaerobic chamber, and then the ingredients were allowed to equilibrate with the internal atmosphere for 2 h before the experiments were started.

The cells were cultured in the presence of 100 μg of streptomycin per ml (after gradual adaptation by increasing the streptomycin concentration weekly in increments of 5 μg ml^{-1}) so as to decrease the risk of contamination during long-term growth periods and experiments. This was particularly helpful against contamination with *Hyphomicrobium* sp.

Analytical procedures. BOD, COD, NH_3, NO_2^-, and NO_3^- concentrations were determined by standard methods (5); and the NH_2OH concentration was determined by the method of Payne (20).

RESULTS AND DISCUSSION

Distribution of nitrifying bacteria in Beer Sheba stabilization ponds. The distribution and concentration of nitrifying bacteria throughout the Beer Sheba wastewater treatment plant was monitored during 1984 by using FITC-conjugated antibodies (Table 2) and by serial dilution. The direct count is a value that represents only those bacteria that reacted with the antibodies prepared against the original isolates, and the possibility that antigenically different strains are misrepresented should not be ignored. When the number of nitrifying bacteria was determined by serial dilutions, however, the corresponding values were somewhat lower and usually did not exceed 10^3 cells ml^{-1}. This discrepancy might result from the presence of dead or nondividing cells. The total number of heterotrophic viable bacteria was also counted routinely on nutrient agar plates and fluctuated between 1×10^7 and 2×10^8 cells per ml.

Continuous culture of nitrifiers on wastewater. A 10-liter chemostat was fed with wastewater drawn from the anaerobic pond, which was characterized by high BOD and a high NH_3 concentration (Table 1). In addition, the feeding water contained ca. 10^2 viable NH_3-oxidizing bacteria per ml. The water was brought once a week and stored at 4°C.

In an attempt to reveal possible correlations between operational parameters of the chemostat and the viability and physiological activity of NH_3 and NO_2^- oxidizers, the chemostat was operated at variable retention times (5 to 200 h) and variable O_2 concentrations (from anaerobic conditions to air saturation).

In Fig. 1 the correlation between O_2 concentration and the number of NH_3 oxidizers is shown. These are the combined data of all retention times above washout rates (>50 h). Only values above 10^3 to 10^5 cells ml^{-1} represent growth, because as mentioned above, feed water contained ca. 10^2 cells of NH_3 oxidizers per ml. These increased in number only at O_2 concentrations of at least 0.1 to 0.2 mg liter^{-1}; the latter figure also appears to be close to the optimal concentration, as a higher concentration did not change significantly the number of NH_3 oxidizers that were present in the chemostat. On the other hand, nitrification itself proceeded at lower O_2 concentrations (Fig. 2). Diurnal cycles of concentrations as low as 0.05 mg of O_2 liter^{-1} for just 6 h and zero concentra-

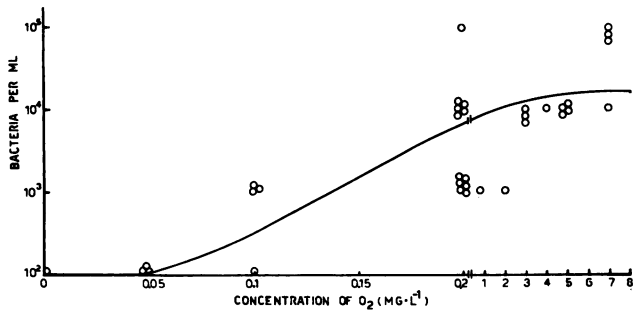

FIG. 1. Oxygen concentration versus number of *Nitrosomonas* sp. cells, as determined by serial dilutions in a chemostat fed by water from an anaerobic pond from 1 April 84 to 2 December 84. The retention time was 100 h. For details, see the text.

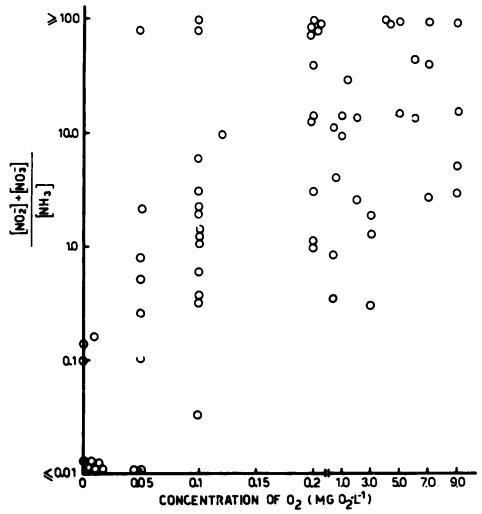

FIG. 2. Combined data of nitrification versus O_2 concentration in the chemostat maintained at various retention times (50 to 150 h). When the chemostat was operated at regulated low O_2 concentrations (0.05 to 0.2 mg liter^{-1}), these values were maintained for only 6 h during the day, with O_2 concentrations being below the detection level (<0.01 mg liter^{-1}) for the rest of the day. Feed water specifications are presented in Table 1 (anaerobic pond). Each point represents the results of one experiment done after the steady state was achieved (3 to 5 days of operation).

tion for 18 h enabled nitrification of ~50% of the NH_3 present in the culture vessel. A change in the O_2 concentration between 0.05 and 1.0 mg of O_2 liter^{-1} at any retention time between 50 and 150 h did not change significantly the rate of nitrification (Fig. 2). Interestingly, the number of nitrifiers in itself was not directly correlated with nitrification rates, and even 10^2 cells ml^{-1} maintained a significant rate of nitrification (Fig. 3).

The field observation data presented above suggest that even a hypertrophic, almost permanently anaerobic biotope, such as an anaerobic stabilization pond, is capable of sustaining a small but viable population of nitrifying bacteria. The chemostat experiments show that even very low oxygen input immediately triggers oxidation of NH_3 by this population.

Ram Reservoir. The Ram Reservoir was studied during 1985 in detail (Table 3) with respect to nitrification.

As the values in Table 3 reveal, during stratification of this reservoir the number of nitrifiers increased with time in the hypolimnion to values much higher than those in the epilimnion. The most dramatic demonstration of this phenomenon was observed on 20 August 1985, when concentrations of *Nitrosomonas* sp. reached 10^6 cells ml^{-1} and *Nitrobacter* sp. reached 10^5 ml^{-1}, which is three orders of magnitude more than their respective counts at the epilimnion.

Maale Kishon Reservoir. The Maale Kishon Reservoir receives good-quality effluents and was monitored as described above for the Ram Reservoir to see whether oxygenation and low organic load increase nitrifying activity. Nitrifying bacteria, ammonia, nitrite, and nitrate concentrations were monitored at the center of the northern part (SR2) and at the outlet of the southern part (SR5) of the reservoir (Table 4).

Although the Maale Kishon Reservoir is well oxygenated and has a low organic load, as can also be judged by the presence of NO_2, NO_3^-, or both in nearly all samples, the concentration of ammonia in the water remained high and the number of nitrifiers was within the distribution range in the epilimnion of Ram Reservoir (Table 3). In Fig. 4 the change in NH_3 concentration in the water along the movement through the reservoir, from the inlet (S5) to the outlet (SR5), is described. In the Maale Kishon Reservoir it takes on the average of 120 days (Y. Eren, personal communication) for the effluents to move from the entrance of the northern reservoir to the outlet of the southern reservoir (calculated as operating in a plug flow mode). The observed reduction of NH_3 concentration during that period was 52.8%, or 0.44% of the initial concentration per day, along the movement of water from the northern inlet to the southern outlet. The calculated daily rates of elimination of NH_3 in the Ram Reservoir for all years that I have records (Table 5) give similar or slightly higher rates.

The Maale Kishon Reservoir probably represents effluents of the highest quality available in Israel. On the same scale, Beer Sheba effluents represent the other extreme, being among worst available effluents. The data presented here suggest that storage of properly treated, as opposed to poorly treated, wastewater is of no advantage with respect to elimination of ammonia and that it does not guarantee its faster elimination in a wastewater reservoir. Although the two reservoirs are so different with respect to the quality of the stored water, this did not affect nitrification rates. However, the question is not how is it that elimination of NH_3 is so fast in the Ram Reservoir, as why is it so slow in the Maale Kishon Reservoir where everything seems to be in favor of fast nitrification. When the same water samples are brought from the field (from both reservoirs) and diluted in the laboratory in mineral medium for counting nitrifiers by serial dilutions, rates of oxidation of NH_3 were regularly in the range of 200 to 300 mg liter^{-1} in the first dilution test tube, during 21 days of incubation, which is at least 1 order of magnitude faster than in the field. Therefore, either some major inhibiting factor exists in the reservoirs, possibly some toxic agent or an antagonistic interaction with other microorganisms that overrides all the other differences between the two ecosystems, or some factor that is vital for these bacteria is missing in this ecosystem. In the laboratory, under defined aerobic growth conditions in a chemostat fed with mineral medium, population densities of up to 10^8 cells ml^{-1} and nitrification rates that are several orders of magni-

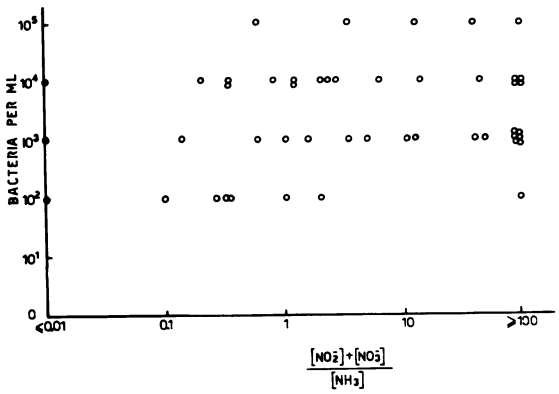

FIG. 3. Number of *Nitrosomonas* sp. cells versus nitrification in chemostat. O_2 concentrations were >0.05 mg of O_2 liter^{-1} for at least 6 h during the day. Retention times were 50 to 150 h.

TABLE 3. Distribution of nitrifying bacteria, BOD, COD, S^{2-} and NH_3 concentrations, and total bacteria counts in Ram Reservoir during 1985

Date	Sampling depth (cm)	Mixing	*Nitrosomonas* sp. (cells ml^{-1})[a]	*Nitrobacter* sp. (cells ml^{-1})[a]	Temp (°C)[b]	S^2 concn (g $liter^{-1}$)[c]	NH_3 concn (mg $liter^{-1}$)	BOD (mg $liter^{-1}$)		COD (mg $liter^{-1}$)		Total bacteria counts (heterotrophic)
								Total	Filtered	Total	Filtered	
20 May	0	Totally mixed	10^1	10^2	18							
	600	Totally mixed	10^2	10^2	18							
4 June	0	Stratified	10^1	10^1	25							
	600		10^2	10^2	20							
18 June	0	Stratified	10^2	10^3	25							
	600		10^3	10^4	20							
8 July	0	Stratified	10^3	10^3	25							
	500		10^3	10^3	20							
31 July	0	Stratified	10^3	10^3	25	5×10^{-4}	20.4	60	20	560	400	
	400		10^4	10^4	20	1×10^{-2}	80.9	130	110	640	500	
20 August	0	Stratified	10^3	10^2	25	5×10^{-8}	20.4	155	120	360	160	
	400		10^6	10^5	20	7×10^{-3}	85.5	170	150	440	240	
22 October	0	Mixed	10^1	10^1	18	1×10^{-4}	61.2	50	30	320	160	8×10^6
	500		10^2	10^2	18	1×10^{-4}	64.6	80	40	400	320	5×10^6

[a] Data are of viable counts by serial dilutions. These values were in close agreement with direct counts with fluorescent antibodies (data not shown).
[b] Measured at 7 a.m.
[c] Measured with a sulfide electrode (15).

tude higher than in the reservoir are obtained, although there is an abundance of NH_3, O_2, PO_4^{3-}, and trace elements in the effluents. Also, reservoir effluents, when fed into the chemostat in the laboratory, enable growth of up to 10^5 cells of *Nitrosomonas* sp. per ml with rapid nitrification rates (Fig. 1 and 3). One possible reason for the differences between field and laboratory growth of nitrifiers when the same effluents are used might be their sensitivity to blue light (10), but it is difficult to assess this effect in the field, particularly in view of the high turbidity of the water in

TABLE 4. Distribution of nitrifying bacteria and concentrations of NO_2^-, NO_3^-, and NH_3 in Maale Kishon Reservoir[a]

Date and sampling site	Depth (cm)	NH_3 concn (mg $liter^{-1}$)	NO_2^- concn (mg $liter^{-1}$)	NO_3^- concn (mg $liter^{-1}$)	*Nitrosomonas* sp. (cells ml^{-1})	*Nitrobacter* sp. (cells ml^{-1})
20 January 85						
SR2[b]	0	35	0.08	0.2	10^1	10^2
	300	36.5	0.012	0.1	10^3	10^2
SR5[b]	0	35.8	0.53	8.0	10^3	10^2
19 March 85						
SR2	0	36.0	0.08	0	10^2	10^3
	500	36.2	0.06	0.25	10^5	10^4
SR5	0	6.8	0.66	6.1	10^2	10^2
	500	13.6	0.70	7.4	10^4	10^4
17 April 85						
SR2	0	35.6	0.14	0.5	10^1	10^2
	500	36.0	0.14	0.3	10^2	10^2
SR5	0	19.0	0.95	9.5	10^2	10^2
	600	19.0	0.95	9.5	10^2	10^3
9 May 85						
SR2	0	50.0	0.13	0.6	10^1	10^2
	600	50.0	0.03	0.3	10^2	10^3
SR5	0	40.8	2.24	7.0	10^3	10^3
12 June 85						
SR2	0	45.0	0.73	0.3	10^1	10^2
	500	45.0	0.21	0.4	10^1	10^2
SR5	0	20.4	11.0	5.0	10^2	10^3
7 August 85						
SR2	0	47.6	1.0	2.0	10^2	10^1
	300	47.6	1.0	1.5	10^2	10^2
SR5	0	19.7	9.1	5.3	10^4	10^4

[a] The Maale Kishon Reservoir was totally mixed throughout the year.
[b] SR2, center of northern part; SR5, outlet from southern part.

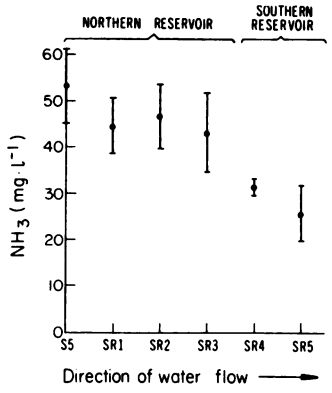

FIG. 4. Change in NH_3 concentration during effluent movement from northern inlet (S5) to southern outlet (SR5) in the Maale Kishon Reservoir. Data were kindly supplied by Y. Eren, Mekoroth Ltd. Values are averages for samples for the period from February to August 1985. Locations: S5, Inlet to the northern reservoir; SR1, 50 m from the inlet; SR2, center of northern reservoir; SR3, connection to southern reservoir; SR4, center of southern reservoir; SR5, outlet from southern reservoir.

question, in which blue light (400 to 430 nm) diminishes at a depth of 10 to 20 cm.

Survival under anaerobic conditions. In view of the observations on the long-term persistence of nitrifying bacteria in the anaerobic hypolimnion of the Ram Reservoir wastewater and the preceding anaerobic ponds, the question arose as to the nature of this long-term survival of *Nitrosomonas* sp. Incubation of *Nitrosomonas* sp. under anaerobic conditions in the standard mineral medium did not support viability much beyond 2 weeks, because between 10 to 17 days its population dropped by 3 orders of magnitude, while the capacity for CO_2 fixation on transfer to aerobic conditions could not be detected after 3 days of incubation under anaerobic conditions in mineral medium. In contrast, the *Nitrosomonas* population in the hypolimnion of the Ram Reservoir (Table 3) during stratification showed a gradual increase from 10^2 cells ml^{-1} on 4 June 1985 to 10^3 cells ml^{-1} on 18 June 1985 and to 10^4 on 31 July 1985 and 10^6 on 20 August 1985. In the permanently oxygenated Maale Kishon Reservoir, the corresponding values were stable within the range of ca. 10^2 to 10^3 cells ml^{-1} (Table 4). At present I do not have any explanation for these observations, but possible clues might be found in reports on several activities of whole cells and extracts of *Nitrosomonas* sp. under anaerobic conditions. Thus, for example, according to Anderson (6, 7), *Nitrosomonas* sp. produces NO from NH_2OH anaerobically in the presence of artificial electron acceptors. Anderson also reported anaerobic uptake of NO in the presence of extracts and mammalian cytochrome *c* (6). I also looked into the possibility of oxidation of NH_2OH under anaerobic conditions by electron acceptors such as PMS, DCPIP, and cytochrome *c*. All were reduced under anaerobic conditions by *Nitrosomonas* sp., while NH_2OH was oxidized. Results of a typical experiment with PMS as an electron acceptor and NH_2OH as an electron donor are shown in Table 6. Hydroxylamine was readily oxidized, but no NO_2^- could be detected under these conditions. Several important controls are also presented in Table 6. Hydrazine is an inhibitor of HAO (16), and in the presence of 1 mM no NH_2OH is oxidized and no PMS is reduced. If NH_2OH is replaced by NH_4OH, no PMS is reduced unless O_2 penetrates the system. Under aerobic conditions and the same experimental setup, the cells reduce PMS in the presence of NH_4OH within seconds. Besides other indicators, this control was also used to check for anaerobiosis during these experiments. Hooper (17) had shown that NO_2^- produced aerobically from NH_3 or NH_2OH has the isotopic oxygen composition of H_2O and that cells of *Nitrosomonas* catalyze rapid exchange with water of both oxygen atoms of nitrite; both observations hint at the possibility that H_2O rather than O_2 is involved in the oxidation of NH_2OH to NO_2^-.

To determine whether there exists a potential for anaerobic activities of *Nitrosomonas* sp. in wastewaters, 1.5 liters of filtered (cellulose acetate; pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) effluents of the anaerobic pond (Table 1) were lyophilized, and the dry powder (1.32 g) was dissolved in 150 ml of distilled water. In a 30% solution of this concentrated effluent 0.135 mM PMS (45%) was reduced by *Nitrosomonas* sp. cells (25 μg of protein ml^{-1}) under anaerobic conditions, while no reduction was observed in the absence of bacteria (Table 7).

The concentration of NH_2OH in these reconstituted effluents was 0.2 μM, which is 3 orders of magnitude less than the amount of PMS reduced in this experiment, and therefore could not be responsible for the reaction. The cells must have used some other substrate for reducing PMS anaerobically.

The capacity of these cells, when they are intact, to use PMS, methylene blue, DCPIP, or cytochrome *c* as electron acceptors under anaerobic conditions is interesting in itself, as these are reduced externally without penetrating into the cytoplasm (11, 18). This reaction does not take place in the periplasmic space, as spheroplasts of *Nitrosomonas* also were found to catalyze oxidation of NH_3 to NO_2^- (8), indicating that the enzymes are bound to the cell membrane or remain in cisternae that are open to the periplasmic space.

Rates of nitrification in the Ram and Maale Kishon reservoirs were very similar, although the Ram Reservoir was highly loaded with organic matter and the Maale Kishon Reservoir received water with a very low organic load and was permanently aerobic. These observations are in accordance with the laboratory observations in which maximal nitrification rates in chemostats fed by anaerobic effluents of the settling pond were obtained at very low oxygen concentrations (0.05 to 0.1 mg $liter^{-1}$). The observation that rates of nitrification of NH_3 in samples brought from the field were much faster, however, requires identification of the responsible factor(s) before any attempt to control or increase nitrification rates in wastewater reservoirs or oxidation ponds can be made. Proliferation of nitrifying bacteria in the hypolimnion of a wastewater reservoir that was stratified for months, when compared with their rapid decline when

TABLE 5. NH_3 concentration in the last settling pond of the Beer Sheba wastewater treatment system and in the wastewater reservoir effluents

Year	NH_3 concn (mg $liter^{-1}$)		Rate of elimination of NH_3/day (% of influent concentration)
	Settling pond	Reservoir	
1980[a]	47 ± 18	25 ± 9	1.2
1981[a]	61 ± 22	37 ± 23	0.98
1982[b]	76 ± 8	53 ± 6	0.76
1985[b]	69 ± 7	57 ± 2	0.42

[a] From previously published data (1).
[b] Unpublished data.

TABLE 6. Oxidation of NH_2OH under anaerobic conditions with PMS as electron acceptor

Time (min)	NH_4OH (2 mM)	NH_2OH (2.2 mM)	PMS (1 mM)	*Nitrosomonas* sp. (6 μg of protein per ml)	Hydrazine (1 mM)	PMS A_{435}	NH_2OH concn (mM) after incubation
0	−	+	+	+	−	2.40	2.2
20	−	+	+	+	+	2.40	2.2
20	−	+	+	+	−	0	1.75
40[a]	−	+	+	+	−	0	1.30
20	−	+	+	−	−	2.40	2.2
20	−	−	+	+	−	2.40	
20	−	+	−	+	−		2.2
20	+	−	+	+	−	2.40	

[a] Another 1 mM of PMS was added at 20 min.

TABLE 7. Reduction of PMS by *Nitrosomonas* sp. with reconstituted effluents[a]

PMS (0.3 mM)	Effluents (1.0 ml)	Cells[b]	Reduced PMS (mM)
+	+	−	0
+	−	+	0
+	+	+	0.135

[a] Incubation time was 4 h; reconstituted effluents were from an anaerobic pond and were concentrated 10 times (final volume, 3 ml).
[b] 25 μg of protein ml^{-1}.

maintained anaerobically in mineral medium in the laboratory, suggests that alternative metabolic pathways are used by these microorganisms in this biotope, not necessarily involving or depending on oxidation of ammonia and nitrite for energy.

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